IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Court et al.
Application No. 10/692,553
Filed: October 23, 2003
Confirmation No. 1179
For: ENHANCED HOMOLOGOUS RECOMBINATION MEDIATED BY LAMBDA RECOMBINATION PROTEINS
Examiner: Jennifer Ann Dunston
Art Unit: 1636
Attorney Reference No. 4239-66898-01

CERTIFICATE OF MAILING

I hereby certify that this paper and the documents referred to as being attached or enclosed herewith are being deposited with the United States Postal Service as First Class Mail in an envelope addressed to: MAIL STOP AMENDMENT COMMISSIONER FOR PATENTS, P.O. BOX 1450, ALEXANDRIA, VA 22313-1450 on the date shown below.

Attorney or Agent for Applicant(s)

Date Mailed August 24, 2006

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DECLARATION UNDER 37 C.F.R. § 1.131

We, Neal Copeland, Daiguan Yu, Hilary M. Ellis, Donald E. Court, E-Chiang Lee, Nancy A. Jenkins, and Pentao Liu, declare as follows:

1. We are the inventors of the above-identified application, which is a continuation of U.S. Patent Application No. 10/366,044, filed February 12, 2003, which is a continuation-in-part of PCT Application No. PCT US01/25507, filed August 14, 2001, which claims the benefit of U.S. Provisional Application No. 60/225, 164, filed August 14, 2000 and claims the benefit of U.S. Provisional Application No. 60/271,632, filed February 21, 2001.

2. It is our understanding that the claims 1, 3, 4 and 13 are rejected as allegedly being anticipated by Cassanova et al., Genesis 32(2): 158-160, published online February 13, 2002.

3. We conceived of, and reduced to practice, a method for generating a vector for conditional knockout of a gene in a cell including a de-repressible promoter operably linked to a

nucleic acid encoding Beta and Exo, as claimed in claims 1, and 2-13, prior to February 13, 2002, in the United States.

3. The methods of claims 1, 3, 4 and 13 were conceived of prior to February 13, 2003. Selection cassettes for use in the claimed methods were made and improved prior to February 13, 2002; some of the experimental work conducted prior to February 13, 2002 is described below. Photocopies of Dr. Liu's laboratory notebook pages, labeled pages 1-10 are submitted herewith. The photocopied pages are referred to below as "the laboratory notes." Dates on these pages have been redacted. Prior to February 13, 2002, we performed the following experiments in the United States, which are documented on the laboratory notebook pages:

We constructed a plasmid that including a selectable marker (specifically a kanmycin/neomycin resistance marker) flanked by a pair of recombining sites (specifically LoxP). This plasmid was designed to introduce the recombining site into a genomic locus on a bacterial artificial chromosome (BAC) or a plasmid. A diagram of this plasmid, and a restriction map of this plasmid is shown in the laboratory notes, see page 1. The selection marker is called PL400.

We also constructed PL428 and PL430 which were additional plasmids for introducing recombining sites (LoxP sites) into the 5' and 3' sides of a genomic fragment of the Ctip2 locus. This is documented in the attached photocopy of Dr. Liu's laboratory notes, labeled page 2. DNA fragments of PL428 and PL430 were restriction digested or amplified by polymerase chain reaction. These fragments, containing the selectable marker (Kan-Neo) flanked by two recombining sites (LoxP) and having homology arms, were electroporated into E. Coli cells containing a de-repressible promoter (pL) operably linked to a nucleic acid encoding Beta and Exo. The production of kanamycin resistant cells is documented at the bottom of page 2 ("Kan^R"). A recombinase (Cre) is used to excise the nucleic acid encoding the selectable marker to leave a single first recombining site in the gene, as indicated on the right side of page 3 of the laboratory notes.

To clone a mouse genomic fragment from a BAC using recombineering, in order to make the conditional targeting vector, a retrieval vector (PL433) was constructed. PL433

includes two short DNA fragments from the end of the genomic DNA fragments. There is a MC1TK (thymidine kinase, a second selectable marker) in the backbone of this plasmid, negative selection could be used in embryonic stem cells with this conditional targeting vector. The production of PL433 is documented on page 4 of the laboratory notes.

The PL433 plasmid was electroported into E. coli cells wherein the de-reprssible promoter was de-repressed. Two colonies were examined by digesting the DNA with restriction enzymes. The restriction pattern documented that the selectable marker (TK) was inserted flanked by a second pair of recombining sites (LoxP). This produced plasmid PL435, shown on page 5 of the laboratory notes, which contained the genomic fragment (Ctip2) for making the targeting vector.

The DNA insert (2.8 kb in length) from PL430, which contained the selection marker (Kan-Neo) flanked by two recombining sites (loxP) was co-electroported into bacterial (E. Coli) cells including a derepressible promoter (pL) operably linked to Gam and Exo. The cells were heat induced to insert the first recombining site into the Ctip2 locus. The correctly targeted plasmid was re-transformed into bacterial cells (E. coli). The loxP-flanked Kan marker was excised in the E. coli to leave a single loxP site in the genomic DNA. (see page 6 of the laboratory notes, top panel). This new plasmid was co-electroporated with the DNA fragment from PL436 containing the Neo-Kan selection maker also flanked by a second pair of LoxP sites. This resulted in the production of plasmid PL437. PL437 is the conditional knock-out vector that will allow deletion of the last exon of Ctip2 (see page 6 of the laboratory notes, bottom panel). The configuration of PL437 as a conditional targeting vector was confirmed using restriction digestion, as shown on page 7 of the laboratory notes.

A vector for conditional knock-out of the Evi9 locus was generated. This conditional targeting vector was designed to delete exon 4 of the Evi9 gene. The construction of this vector is shown on page 8 of the laboratory notes.

PL438 was a plasmid that contained a first pair of recombining sites (two LoxP sites, also called "floxed") flanking a selection marker (Neo-Kan), and flanked by two PCR amplified genomic DNA fragments. These genomic fragments could be used as homology arms in recombineering. The insert from this plasmid placed the floxed selection marker (Kan) into the 5' side of exon 4 (within exon 3) of the Evi9 gene. This plasmid could be used to introduce the first recombining sites into a BAC.

PL440 was a plasmid also contained a pair of recombining sites (LoxP or "floxed") flanking a selection marker (Neo-Kan) and flanked by a two polymerase chain reaction (PCR) amplified genomic DNA fragments. PL440 was of uses for recombineering. The insert from PL440 was used to place a floxed selection marker (Kan) into the 3' region of exon 4 (in intron 4) of the Evi9 gene. This plasmid could be used to introducing the second pair of recombining sites into a BAC.

PL441 was then constructed. This is a retrieval vector for retrieving the Evi9 genomic DNA fragment from an Evi9 BAC (see the bottom of page 8 of the laboratory notes). Linearized PL441 was electroported into an Evi9 BAC (called "C3," see page 9 of the laboratory notes). The retrieved plasmid was called PL442. PL442 was co-electroporated with the insert from PL438 to place a floxed Neo-Kan selectable marker into intron 3 of Evi9 (see page 9 of the laboratory notes).

The targeted plasmid was transformed into E. coli expressing a recombinase ("Cre") to excise the selectable marker. This left a single LoxP site in intron 3 of Evi9. The production of this allele is shown in the top panel on page 10 of the laboratory notes.

The excised plasmid was then co-electroporated with the insert from PL440 to place a second floxed selectable marker (Neo-Kan) into intron 4 of Evi9. Thus, the plasmid PL443 was produced, which is a conditional targeting vector that could be used to delete exon 4 (located between intron 3 and intron 4) of Evi9. The production of PL443 is shown in the bottom panels on page 10 of the laboratory notes. We were aware that an Frt site could be used as a recombining site in the place of a loxP site, and that Flp could be used as the recombinase. A strain of *E. Coli*, EL250 was created that expresses Flp.

4. These results demonstrated: (1) homologous recombination could be used to insert a nucleic acid encoding a selectable marker (Neo-Kan) flanked by a pair of first recombining sites (LoxP) into a first site (one intron) in a gene (Evi9 or Ctip2) in vector including bacterial artificial chromosome (Evi9 or Ctip2), (2) homologous recombination could be used to insert a nucleic acid encoding a selectable marker (Neo-Kan) flanked by a pair of second recombining sites (LoxP) and a first recombining site into a second site (a second intron) in the gene (Evi9); (3) the nucleic acid encoding the selectable marker could be excised with a first recombinase specific (Cre) specific for the recombining sites, leaving a single first

recombining site in the gene (Evi9 or Ctip2), and (4) the nucleic acid encoding a selectable marker (Kan-Neo) could be excised with a recombinase (Cre) specific for the second recombining sites. Two recombining sites remained in the gene following excision of the nucleic acid encoding the selectable marker, thus generating a vector for conditional knockout of the gene (Evi9 or Ctip2). *E. coli* strains were created that expressed Flp, so that Frt recombining used. The homologous recombination was performed in bacterial cells including a de-repressible promoter (pL) operably linked to a nucleic encoding Beta and Exo.

5. All statements made herein and of our own knowledge are true and all statements made on information are believed to be true; and further, these statements were made with the knowledge that willful false statements and like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements made may jeopardize the validity of the application or any patent issuing thereon.

Date _____ _____
Neal G. Copeland

Date 7/26/2006 Daiguan Yu
Daiguan Yu

Date _____ _____
Hilary M. Ellis

Date _____ _____
Donald L. Court

Date _____ _____
E-Chiang Lee

Date _____ _____
Nancy A. Jenkins

Date _____ _____
Pentao Liu

SAS:sas 08/17/06 555051.doc E-177-2000/2-US-02

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Date Mailed ~~July 13, 2006~~ August 21

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Date _____ Neal G. Copeland

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Date _____ Hilary M. Ellis

Date _____ Donald L. Court

Date _____ E-Chiang Lee

Date _____ Nancy A. Jenkins

Date August 16, 2006 Pentao Liu

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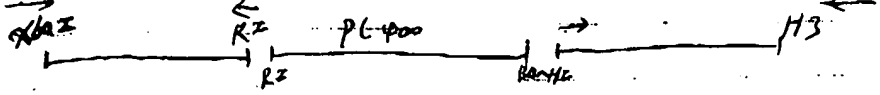
PCR fragments for making targeting vector to introduce 1st 6xP.

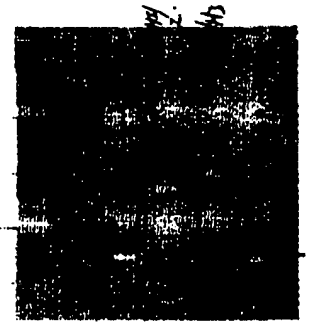
① XbaI/RI XbaI - 10966 Ctip², RI - 11274 Ctip²

② BamHI - H3 BamHI - 11275 Ctip², H3 - 11532 Ctip²



2λ	XbaI/RI	0
2λ	PL400	2
2λ	BamHI/H3	0
2λ	PSKF	2
1λ	10 x buffer	1
1λ	T4 ligase	1
		6 tube

1λ P-A 2
1λ dNTP
1λ prim 1
1λ P-2
1λ H2O 4L

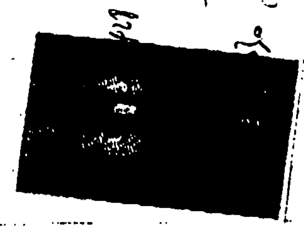


XbaI/XhoI
XhoI/H3
XbaI/RI/H3

digest pL428 #16 pL430, #2, #4 w/ Not2/Sal2/Pvu2
to excise the targeting cassettes.

15λ plasmid
4λ buffers
2λ enzymes (Not2, Sal2, Pvu2)
20λ H_2O
42λ

37°C 1.5 hr. gel purify



spin column gel purify
↓ QIAquick
30λ 20ng/λ

use 3λ + 1λ pL424 (110ng) #27.
electroporation Time constant 4.4
so can use more if needed.

42°C → 240 kan^r ←
32°C → 4

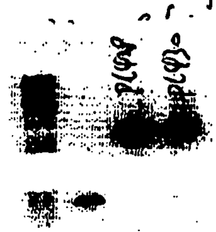
Can also amplify the whole-targeting vectors by PCR

① amplify pL428 w/ XbaI-13264-cfip2
H3-13833-cfip2

② pL430 w/ XbaI-10966-cfip2
H3-1

use Not2/Sal2/Pvu2 cut

PCR products were purified by QIAquick → 28λ EB
pL428: 360ng/λ
pL430: 330ng/λ



1λ DNA
1λ dNTP
1λ primer1
1λ primer2
21λ H_2O
5λ 2
0.45 Taq
20λ

use 1λ pL424 #27 (110ng)
plus 1λ pL430 330ng or 2λ pL430 660ng
↓ 168 Kan^r ↓ 176 Kan^r

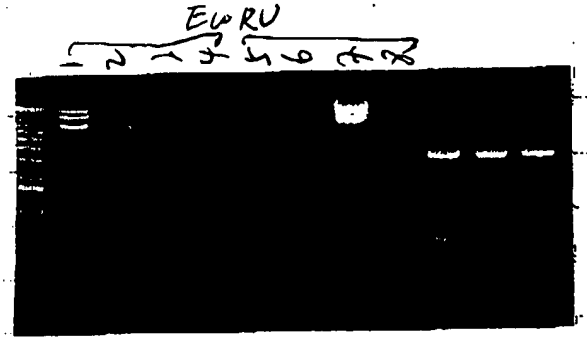
if use 32°C cells ↓ 0 Kan^r

seems increased targeting DNA does not increase Kan^r
might need more target DNA (pL424_#27)
↓
miniprep.

#1 → #4 ... from targetfy ω purified plasmid insert pL4530

#5 → #8 ... from ... ω PCR of pL4530

~~plasmid~~ ~~or~~ insert from plasmid is better.



use #2 electrop → E1350 → Kan^r

#2 → CVE induced E1350 → Kan^r / Amp^r

#1, #2 from

#3, #4 - still mixed after popout

use #4 mixed-popout cells directly and electroporated ω pL428 purified cassette 42°C induced. no Kan colony. indicating e- electroporation is a must

primers

① NotI-cfip2-ret-5'-1-4001
H3-cfip2-ret-5'-2-4371

② H3-cfip2-ret-3'-1-17779
SpeI-cfip2-ret-3'-2-18145

DNA
C5

③ BamHI-CG9650-5'
④ XhoI-CG9650-3'
⑤ Same as ③

Fly adult RT Random primer

Fly adult RT oligodT priming

⑤ BamH2-CG9650-3
XhoI-CG9650-3'

Fly adult RT Random primer

⑥ Same as ⑤

Fly adult RT oligodT priming.

#1, #2 work
#3 → #6 do not work.

#1, #2 were purified w/ QIAquick and digested w/ appropriate enzymes, and again purified w/ QQ.

1% agarose gel.

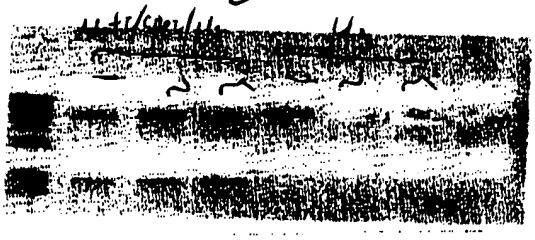
#1 cut w/ Not2/H3, #2 cut w/ H3/Spe2, P(25)2 Not2/Spe2 ligation.

3λ #1
3λ #2
2λ P(25)
1λ 10× Lig
1λ T4 ligase

1λ DNA
1λ dNTP
1λ primer1
1λ primer
21λ H2O

5λ 10×
0.75λ Taq
20λ H2O

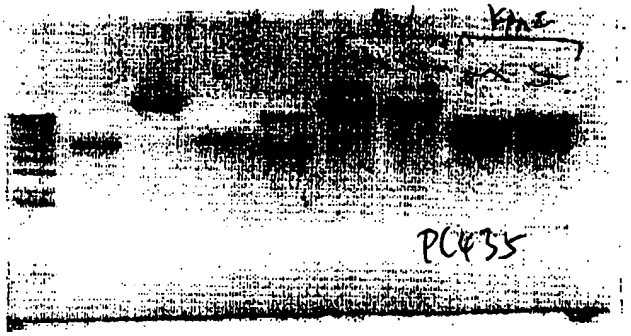
pL433



(4)

retrieving ... pC433 (130ng/λ) with λ
(42°C induced chip: C5 BAC
↓
Amp plate

total colonies: 42°C 1200 × 8 = 9600
32°C 11 × 8 = 88



PL435 co-electroporation w/ PL430 insert to target the 1st loxP to the cfip2 retrieved plasmid.

9AM. inoculate EL350

42°C 120 colonies, 32°C 10 colonies.

pick up ~~two~~ four. 2 are corrected, #3, #4.

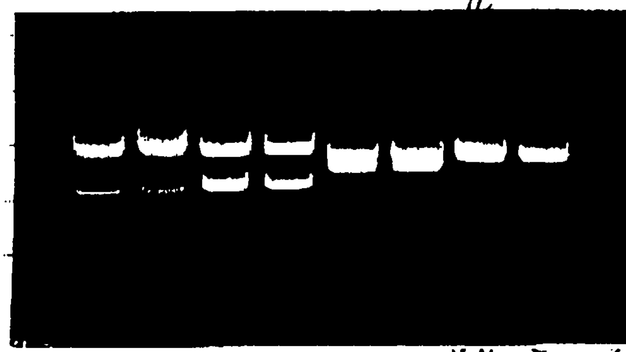
#4 dilute 100x

→ EL350 (cre) frozen to pop out

→ DH10B

		wt.
BamHI	14.5 ~~12~~ kb / 7 ~~8~~ kb	19 kb
RV	11 kb / 10 kb	~~10 kb~~ 9 kb

no mixture of untargeted plasmid!



EL350 (popout) → 100x ~~plating~~ ~~300 colonies~~. DH10B thousands need dilution in ~~???~~ Amp

9AM. inoculate 5ml EL350

directly from pop out colonies. inoculate two → ~~grow~~ grow 10hrs → miniprep

~~①~~ one has 50ng/λ, 2nd 110ng/λ DNA

use the 2nd one 1λ + 1λ PL436 cassette (150ng/λ)

↓

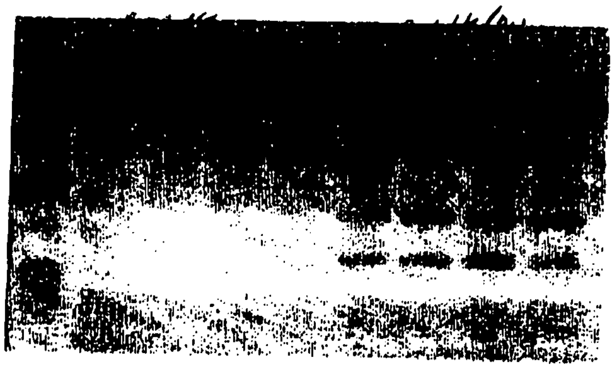
42°C induced EL350.

42°C 102 colonies

32°C 9 colonies. pick up 4 → 32°C → o/n

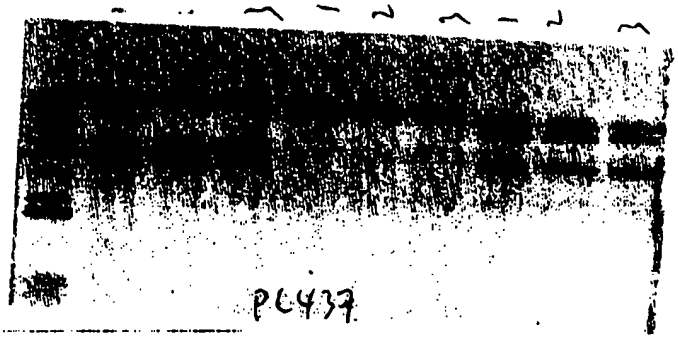
#2, 3, 4 are mixed w/ targeted and non-targeted

take 1ng of #4 → DH10B

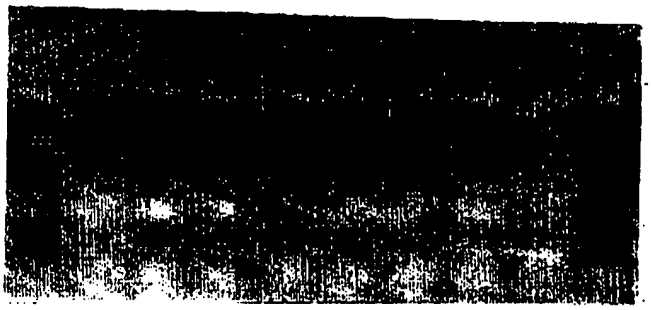


PL437

Minipreps 3 colonies of DH10B



cfp1 Conditional targeting Vector



Evi9 exon 4 retriv & 3' loxP PCR

primers — DNA

1. NotI - EVI9 - exon 4 - retrve 5'-1 / H3 - Evi9 - exon 4 - retre 5'-2 — { BAC C3 / A5

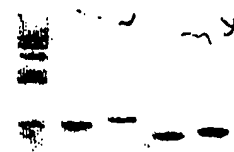
2. H3 - EVI9 - exon 4 - retr 3'-1 / H3 SpeI - Evi9 - exon 4 - retr 3'-2 — { Same

3. XbaI - Evi9 - exon 4 - 3'-L1 / RI - Evi9 - ex. 4, 3'-L2 — { "

4. BamHI - Evi9 - exon 4 - 3'-R1 / H3 - Evi9 - exon 4 - 3'-R2 — { "

(margin note: introduced loxP 3' primer ... enzyme sites ... for cap's)

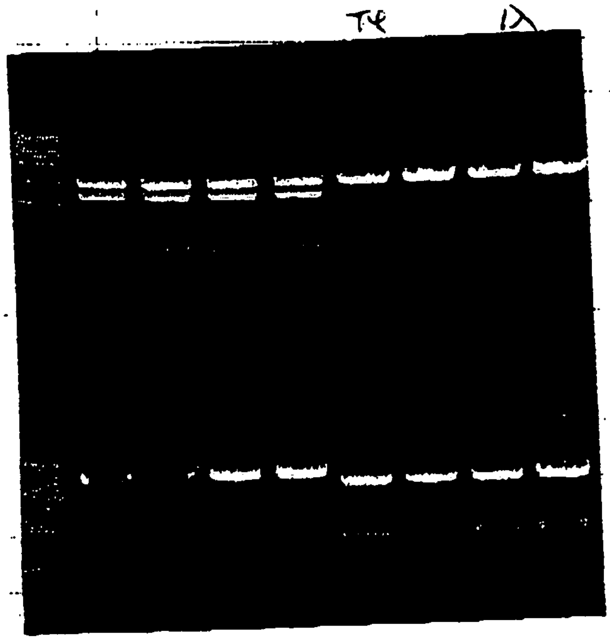
1λ	DNA	4
1λ	dNTP	4
1λ	prim 1	
1λ	prim 2	
21λ	H2O	84
5λ	2	20
0.75λ	λ	3
20λ		Pfu



→ purify w. columns and cut. → purify

1,	3λ
2,	3λ
PL453 NotI/SpeI	2λ
10x	1λ
T4	1λ

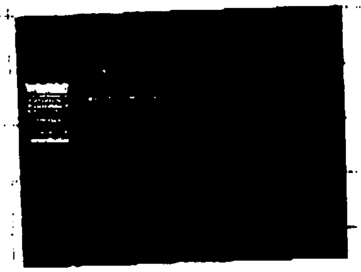
3,	28λ
4,	28λ
PL400 Bam/RI	3λ
PSK+ XbaI/H3	2λ
10x	1λ
T4	1λ

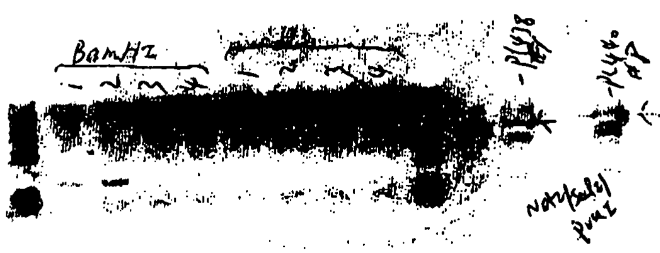


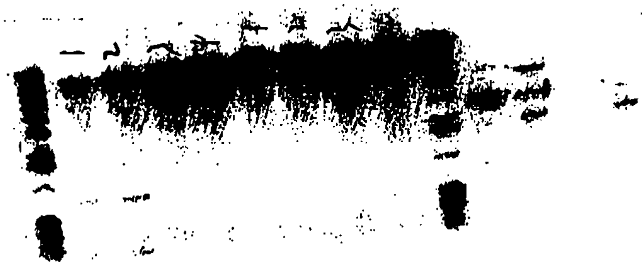
PL440: loxp to 3' of Evi9, exon 4

PL441. retriving vector for exon 4, Evi9

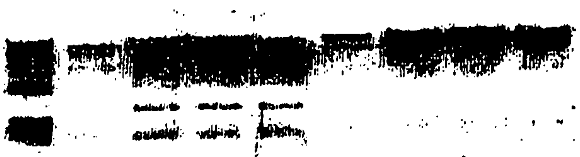
Ev's BAC A5. C3 electroporated to EL350 ≈ 300-400 Colonies
electroporated H3 cut pL441 → A5. C3 BACs
thousands Amp^r colonies recovered
pick 4 2 (#1, #2) from A5, 2 (#3, #4) from C3
10 hours at 32°C w Amp. miniprep
Only #4 (maybe A3) is correct, indicating A5 BAC may not have exon 4.
take 2λ #4 #2 → 200λ TE. take 1λ → DH10B (7λ) in LB
plate 100λ, 10³ colonies grow up. pick
#1 – #2. original #4 prep
#3 – #4 original #2 prep (in-correct).

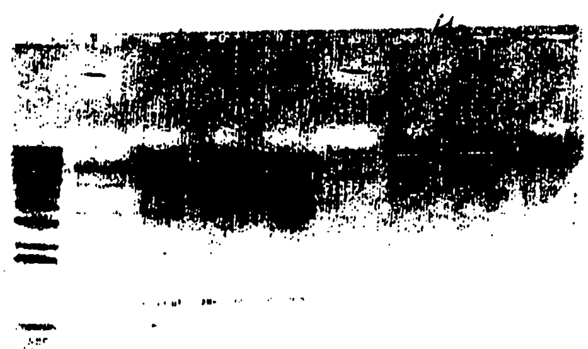






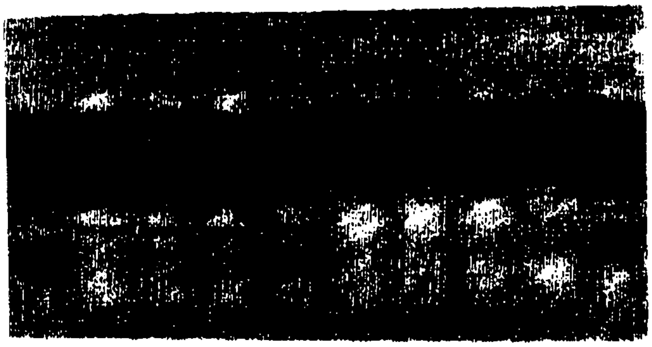
pL438 #1, NotI/SalI → 10ng/λ 3λ each electroporate → EL350 42°C
pL442 #2 → 10ng/λ 0.5λ each
plate on wrong antibiotics (cm), re-do
re purify pL438 #1, 50ng/λ. use 2λ. use 75ng pL442 #2 → EL350
1350 … 1 ml → 10 ml, 2 hr 32°C 20' at 32°C incubator (waiting for water bath to 42°C) 42°C → 500 Kan → 120 Kan pick up 4





all are targeted
but #4 do not have WT contamination
→ used for popout

EVI9 exon4 conditional allele . 1st Cosp popout



1-1-02. targeting the popout plasmid #2 (75ng) w/ pL440 insert (100ng) using the frozen EL350 competent cells → ≈44/45 = 200 colonies

2nd 10x PSA pick up 4 colonies. 3 targeted. Transform #2 → DH10B Kana plate.

pL443